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Preview

Hypoxia-inducible CAR expression: An answer to the on-target/off-tumor dilemma?

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On-target/off-tumor toxicity is one of the major concerns regarding CAR T-cell therapy. Kosti et al.[1] demonstrate that this form of toxicity can be prevented by designing a CAR whose expression is controlled by oxygen levels in the tumor environment.

T cells engineered to express a chimeric antigen receptor (CAR) specific for a tumor-associated antigen (TAA) is a greatly promising therapeutic option for solid tumors. The first step in designing a new CAR is the identification of which antigen to target. The ideal CAR target is defined by high expression on tumor cells and no expression on normal tissues. Unfortunately, such targets are rare, leading many investigators to focus on targets that are expressed to some degree on normal tissues. This raises the possibility of CAR T cell treatment related on-target/off-tumor toxicities. To address this issue, CAR T cell biologists are developing inducible CAR systems where CAR effector function can be controlled. The goal of these approaches is to induce and/or activate CAR expression/function only in the tumor milieu. For example, the SynNotch CAR system requires the presence of two antigens to initiate T cell cytotoxicity, thus reducing the likelihood that normal tissues will be affected.[2] Tet-on systems, in which CAR expression is controlled by the administration of doxycycline, have also been investigated to limit on-target/off-tumor toxicity.[3] However, one limitation of such systems is that their transcriptional regulation relies on non-human proteins, raising questions of immunogenicity. Another approach has been to engineer T cells that recognize molecular signatures consistent with the tumor microenvironment and are hypofunctional in other contexts. For example, Sukumaran and colleagues engineered a three-receptor system in which the presence of a TAA, transforming growth factor β (TGF-β), and interleukin-4 (IL-4) were necessary to provide CD3ζ activation, 4-1BB co-stimulation, and IL-7 cytokine support.[4]

In a current study published in the *Cell Reports Medicine*, Kosti et al.[1] characterize a CAR that is only expressed and functional in hypoxic (low oxygen) environments, which is a hallmark of solid tumors.[5,6] To demonstrate that this system can prevent on-target/off-tumor toxicities, the authors used a CAR that recognizes ErbB family receptors that are highly expressed on tumors but also on normal epithelial cells. This pan-ErbB CAR, named T4-CAR, recognizes 8 out of 9 of the possible ErbB receptor homo- and hetero-dimers and was co-expressed with a chimeric cytokine receptor (4αβ) that delivers an intracellular IL-2/IL-15 signal upon binding of IL-4. While T4-CAR T cells effectively eradicate tumors *in vitro*, rapid-onset lethal toxicity was observed when injected in tumor-bearing mice because of on-target/off-tumor effects, with high levels of T cell accumulation in the lungs and liver. Thus, the authors generated a stringent dual-oxygen sensing system where T4-CAR expression is regulated by hypoxic conditions (Figure 1, "hypoxiCAR"). The promoter of the T4-CAR was modified to incorporate 9 hypoxia-responsive elements (HREs) to allow for HIF1α-dependent transcription in low oxygen conditions. In addition, the T4-CAR sequence was fused to the oxygen-dependent degradation domain (ODD) of HIF1α,[7] which is ubiquitinated in normoxic conditions and directs the hypoxiCAR to the proteasome for degradation. This two-component system therefore provides positive regulation in hypoxic conditions (via HIF1α-mediated transcription) and negative regulation in normoxic conditions (via ODD ubiquitination and CAR degradation). It is important to note that a hypoxia-inducible CAR system has been previously reported.[8] However, the previously reported system only contained negative regulation via the ODD domain and had limited activity *in vivo*. In the present study, the authors improved the hypoxia sensing CAR system and made it more stringent.

Using the dual-oxygen-sensing system, the hypoxiCAR was shown to exhibit strict oxygen-dependent expression both *in vitro* and, more importantly, *in vivo*, where T cells recovered from the tumor had high CAR expression, but those recovered from the blood, lungs, and liver did not have any detectable CAR expression. Furthermore, the hypoxiCAR provided potent anti-tumor activity without any evidence of on-target/off-tumor toxicity in HN3 (head and neck cancer) and SKOV3 (ovarian cancer) subcutaneous tumor models. This stood in stark contrast to the constitutively expressed T4 CAR that had such extreme side effects the mice reached their humane endpoints at 28 h post-T cell infusion. The authors also showed that hypoxiCAR T cell function is not affected by extreme levels of hypoxia, addressing the concern of hypoxia-mediated immune suppression. Taken together, their data demonstrate that by incorporating a hypoxia-inducible CAR expression system, antigens that are expressed on both tumors and healthy tissue can be safely targeted without on-target/off-tumor toxicity.

The authors also provide data and insights on how patient cohorts could be identified that are most likely to benefit

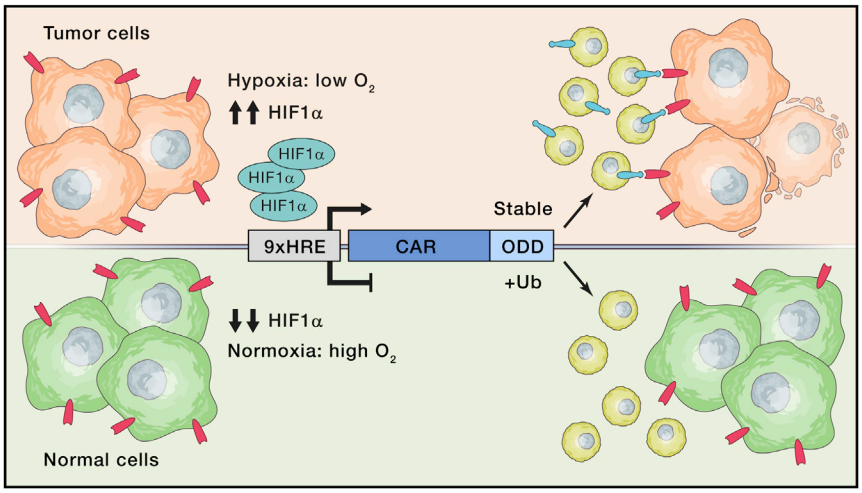

Figure 1. HypoxiCAR expression is tightly controlled by the amount of oxygen in the environment

Top: under hypoxic conditions, HIF1α binds to hypoxia response elements (HREs) within the promoter, increasing CAR transcription. Bottom: under normoxic conditions, the oxygen-dependent degradation domain (ODD) attached to the C terminus of the CAR is ubiquitinated, directing the CAR to the proteasome for degradation.

from the hypoxiCAR. They defined 5 signature genes (PGK1, SLC2A1, CA9, ALDOA, and VEGFA) of which expression in tumors can serve as eligibility criteria for hypoxiCAR study. In addition, IHC of HIF1α and CD3 can also serve as biomarkers for hypoxic tumor environment. While further validation is undoubtedly required, this is an admirable attempt to not only establish a safe CAR system, but also consider factors to guide patient selection in a clinical setting.

While the study is exciting and shows promising results, there are some questions that remain to be addressed in the future. For example, this study only evaluated one CAR, and it would be useful to see whether the system works and prevents toxicities associated with other targets such as GD2, which has been associated with on-target/off-tumor toxicity.[9,10] Also, in-depth studies using immune competent animal models are warranted to fully vet the safety of such a system. Finally, the study only incorporated subcutaneous tumor models, and it remains to be seen whether the hypoxiCAR works as well in more clinically relevant orthotopic tumor models or against metastatic disease.

In summary, the study by Kosti et al.[1] demonstrates that by using a stringent dual-oxygen sensing system, it is possible to overcome the treatment-related toxicities caused by on-target/off-tumor effects. While additional preclinical testing is warranted, this hypoxia-inducible CAR expression system has the potential to greatly expand the array of targetable antigens for CAR T cell therapy.

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